Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors

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To evaluate the potential for adenovirus-mediated central nervous system (CNS) gene transfer, the replication deficient recombinant adenovirus vectors Ad.RSVβgal (coding for β-galactosidase) and Ad-α1AT (coding for human α1-antitrypsin) were administered to the lateral ventricle of rats. Ad.RSVβgal transferred β-galactosidase to ependymal cells lining the ventricles whereas Ad-α1AT mediated α1-antitrypsin secretion into the cerebral spinal fluid for 1 week. These observations, together with β-galactosidase activity in the globus pallidus and substantia nigra following stereotactic administration of Ad.RSVβgal to the globus pallidus, suggest that adenovirus vectors will be useful for CNS gene therapy.

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Rapid progress has been made in the past decade in the identification and characterization of neuroactive proteins and peptides, including neuro-transmitters, neuromodulators, neurohormones and neurotrophic factors, thus opening a variety of new therapeutic possibilities for disorders of the central nervous system (CNS)[1,2]. Although the technology is available to produce these proteins/peptides, delivery of these macromolecules to the brain is markedly limited by the blood-brain barrier, a physical hurdle to the diffusion of molecules from the circulation imposed by the biology of the capillaries of the CNS[1,3]. While this barrier represents a problem for the delivery of many therapeutic agents, it makes the CNS almost inaccessible to circulating proteins and peptides[1,4].

An alternative is to deliver the therapeutic protein/peptide directly to the CNS, either into the substance of the brain or into the cerebral spinal fluid (CSF)[1,4]. Direct delivery to the CSF is an attractive strategy, as it is less invasive and it has the potential to obtain therapeutic levels of the macromolecule over a broad area of the CNS[1,4]. Furthermore, the CSF provides access to the brain by means of the Virchow-Robbins spaces, and macromolecules can readily move from the CSF to the brain via the brain extracellular fluid[1,4,5].

One approach to deliver proteins/peptides to the CSF is through gene therapy, a strategy where the gene or cDNA for the protein/peptide is transferred to the brain, either in cells manipulated *ex vivo* and then implanted in the CNS, or by direct transfer of the gene to the CNS[6–16]. Theoretically, the *in vivo* gene transfer approach is very appealing, as it would permit the use of the gene/cDNA as a "drug", obviating concerns about reactions to the cell delivery system and the cumbersome aspect of carrying out *ex vivo* gene transfer. Direct *in vivo* CNS gene transfer has been evaluated in experimental animals using plasmid-liposome complexes, herpes virus vectors, grafts of genetically modified cells, and transplants of cells producing retrovirus vectors[6–16].

We have investigated a new strategy for gene delivery to the CNS — the use of replication deficient recombinant adenovirus vectors to transfer genes directly to the ependymal cells lining the ventricles, thus establishing a cellular site to produce proteins/peptides that could be secreted directly into the CSF. Our decision to use adenovirus vectors is based on: (i) the ability of adenovirus vectors to transfer genes efficiently to differentiated cells of a variety of organs of adult animals *in vivo*[17–20]; and (ii) the ependymal cells of the CNS posess features of epithelial cells, and adenoviruses have a natural tropism for cells of epithelial origin[17–22]. We have used the adenovirus vector Ad.RSVβgal[19,20] containing the *E. coli lacZ* [β-galactosidase (βgal)] gene as an intracellular reporter gene, and the vector Ad-α1AT[17] containing the human α1-antitrypsin (α1AT) cDNA as an example of a typical secreted glycoprotein. We show that adenovirus vectors can effectively transfer genes to ependymal cells *in vivo*, and provide macromolecules to the CSF for several days following administration of the vector. Unexpectedly, we have also observed that adenovirus vectors can transfer genes directly to parenchymal cells of the CNS, thus broadening the potential applications of this vector system for CNS gene therapy.

In vivo intraventricular administration of Ad.RSVβgal. After intraventricular administration of Ad.RSVβgal, the presence of βgal activity was evident in the region lining

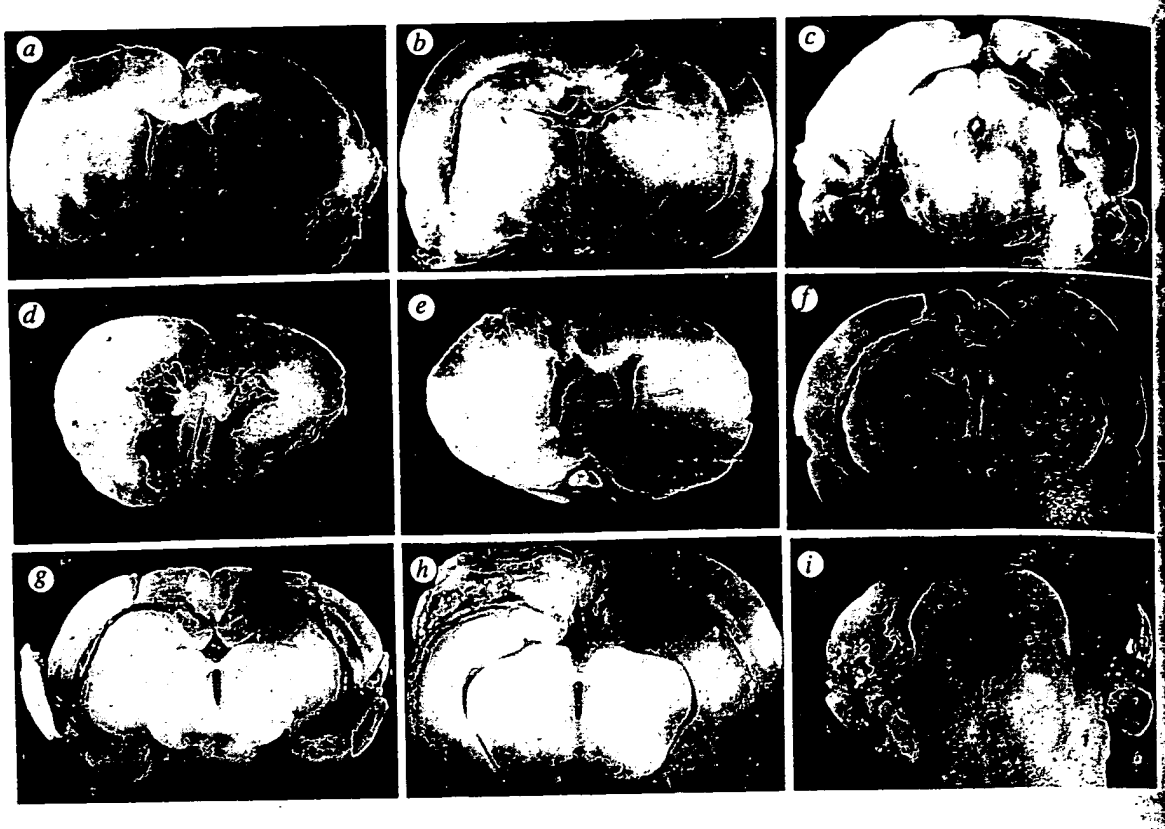
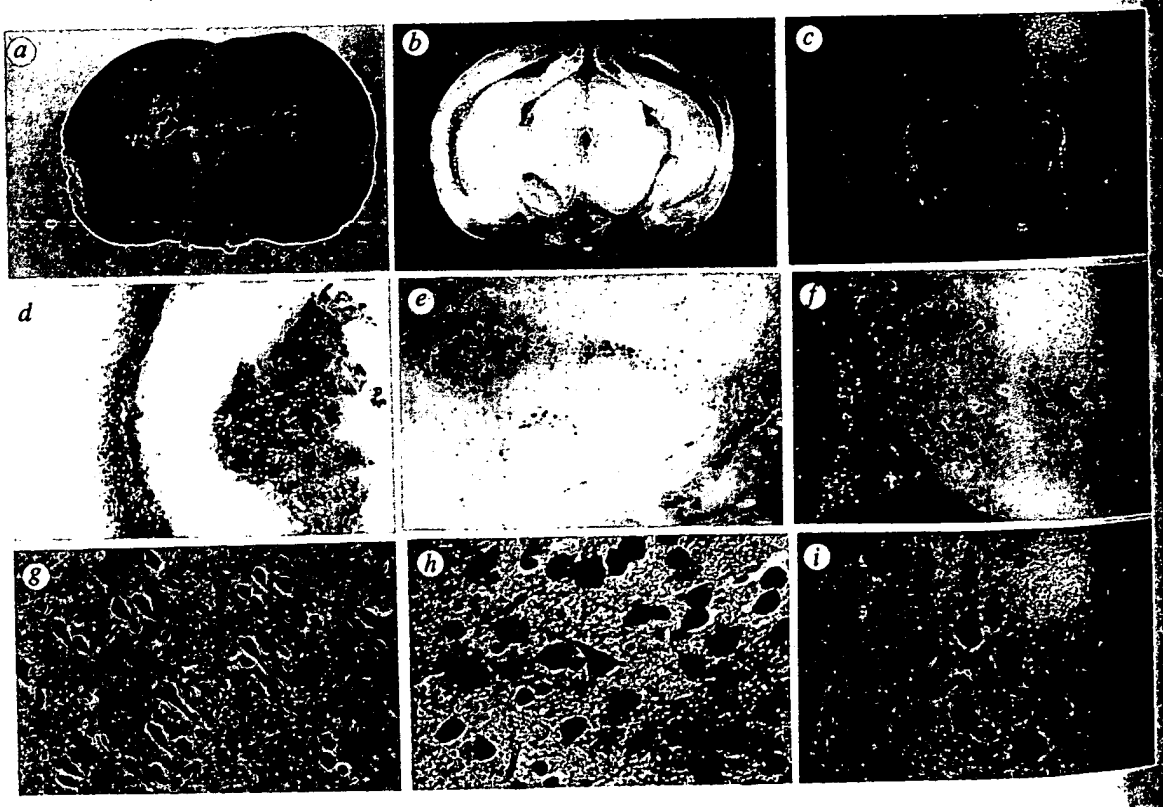


Fig. 1 Expression of the β-galactosidase activity in the ependymal layer lining the ventricles of the rat brain following *in vivo* administration of Ad.RSVβgal via a ventricular catheter. The brains were removed 4 days after administration of the vector and stained with X-Gal reagent; the blue colour indicates β-galactosidase activity. *a-c*, Coronal brain sections at different levels (anterior to posterior) of a rat receiving Ad-α1AT, as a negative control. *d-i*, Coronal brain sections at different levels (anterior to posterior) of an animal receiving Ad.RSVβgal. Note the blue colour lining the ventricular spaces (*d-h*) and on the leptomeningeal surface of the base of the brain (*i*).



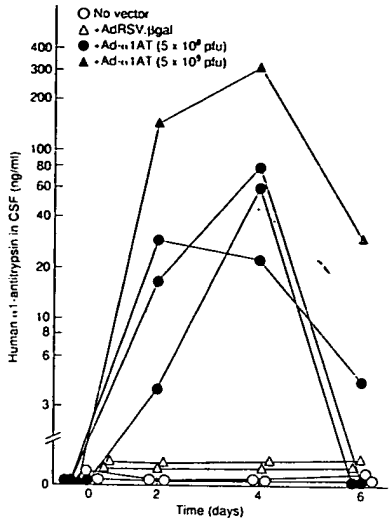


Fig. 3 Human α1-antitrypsin (α1AT) levels in rat CSF sampled in the fourth ventricle at various times after administration of Ad-α1AT into the left lateral ventricle. Human α1AT levels in CSF were quantified using a human α1AT-specific ELISA with a sensitivity of ≥ 3 ng ml^{-1}. The data for different time points from the same animal are connected. Shown is data for Ad-α1AT (• 5×10^8 pfu/animal; ▲ 5×10^9 pfu/animal), and control animals receiving no vector (O) and those infected with Ad.RSVβgal (Δ).

the ventricular system (Fig. 1). The brains of animals that received intraventricular PBS (n=3; not shown) or the control virus Ad-α1AT (n=6, Fig. 1*a-c*) had no blue staining. In contrast, the region lining the ventricles of brains of animals receiving intraventricular Ad.RSVβgal (n=15) appeared intensely blue, indicating a high level of βgal activity (*d-i*). This was true in the lateral ventricle (*d-h*), third ventricle (*g,h*) and fourth ventricle (*i*). The βgal activity was not distributed homogeneously in all ventricles; for example, the dorsal and pineal recess of the third ventricle were negative (*g,h*). Interestingly, the leptomeninges were also positive (*i*), suggesting the Ad.RSVβgal vector administrated to the lateral ventricle was broadly circulated by the CSF.

Microscopic examination of the brain following intraventricular administration of PBS (not shown) or Ad-α1AT, revealed no βgal activity in the ependymal cells lining the ventricles or in cells comprising the substance of the brain (Fig. 2*a,b*). But following intraventricular administration of Ad.RSVβgal, intracellular βgal activity was localized primarily in the ependymal cells lining the cerebral ventricles (*c-i*). This was true for ependymal cells of the lateral ventricles (*c,d*), third (*e,g-i*) and fourth ventricle (*f*). Interestingly, most cells of the choroid plexus did not express βgal after Ad.RSVβgal administration (*c,e*).

***In vivo* administration of adenovirus containing α1-antitrypsin gene.** No human α1AT was detectable in the CSF of injected animals prior to intraventricular administration of Ad-α1AT (n=8) (Fig. 3). Human α1AT levels in CSF were also undetectable after administration of PBS (n=2) or at any time after Ad.RSVβgal administration (n=2). In contrast, human α1AT was easily detected in the CSF of animals 2 days after administration of Ad-α1AT (n=4), with peak levels at 2–4 days, and human α1AT detectable up to 6 days. When a 10-fold higher dose was administrated, the peak levels were severalfold higher. In contrast to the administration of Ad-α1AT, where human α1AT levels remained stable in the CSF for at least 48 h, evaluation of purified human α1AT administered into the lateral ventricle demonstrated a rapid turnover of the CSF, with the amount of human α1AT detectable in the fourth ventricle decreasing rapidly such that levels were only 3% of the initial value (10 min after administration) after 24 h (not shown).

***In vivo* intracerebral administration of adenovirus.** Interestingly, the brains of animals receiving Ad.RSVβgal by stereotactic intracerebral injection into the globus pallidus and part of the striatum area appeared intensely blue, with βgal activity in the region of the injection site and in the ventricular system (Fig. 4*a,d*). In contrast, at the contralateral site, where PBS was administered as a control, there was no blue staining (*a*). Furthermore, βgal activity was detected on the right side (the side of administration of Ad.RSVβgal) in areas of the brain posterior to the site of the injection (*b,c,e,f*), suggesting that there was expression of βgal activity extending from the region of the globus pallidus and striatum to the substantia nigra and/or movement of the Ad.RSVβgal vector (either within neurons or extracellular to the neurons). Histologic analysis of these regions showed that after administration of PBS, no βgal activity was noted in parenchymal cells at the site of injection (*g*). In contrast, following Ad.RSVβgal stereotactic intracerebral administration to the region of the globus pallidus and part of the striatum, the cells in the local site expressed intracellular βgal activity (*h,i*).

◀ Fig. 4 β-galactosidase activity in the brain following intracerebral stereotactic administration of Ad.RSVβgal into the right globus pallidus and in part of the striatum. As a control, phosphate buffered saline was administered to the contralateral region. *a*, Coronal section in the plane of the injection site. The area of the right globus pallidus and striatum shows β-galactosidase activity. *b,c*, Coronal sections (moving posterior, respectively) showing β-galactosidase activity on the right along the tract of nigrostriatal pathway toward the substantia nigra. *d-f*, Closer view of the positive regions showed in *a-c*, respectively. *g*, Site of administration on the control side, 400×. *h,i*, Site of administration of Ad.RSVβgal, 630×.

Discussion

The delivery of potentially therapeutic proteins and peptides to the CNS is limited by the blood-brain barrier[1,3-5]. We have administered replication-deficient recombinant adenovirus vectors directly to the CSF of the ventricular system, targeting the ependymal cell lining the cerebral ventricles. We find that adenovirus-mediated gene transfer to ependymal cells is efficient, and permits expression of proteins either localized in the ependymal cells or secreted into the CSF for several days.

The ependymal cells lining the ventricles are a particularly useful target for CNS directed gene transfer where the goal is to deliver proteins/peptides to the brain. These cells form a single layer, uniformly distributed over the surface of the lateral, third and fourth ventricles, and the central canal of the spinal cord, a surface that is on the CNS side of the blood-brain barrier[23]. Although it is not trivial to administer materials to the CSF, it is much less

invasive than direct administration to the parenchyma of the brain. Furthermore, the anatomic location of the ependymal layer permits secretion of proteins/peptides to the CSF and thus indirectly, to the brain.

One advantage of *in vivo* gene transfer to the ependymal layer over direct administration of the gene product to the CNS is that gene transfer provides a local site of production of the gene product. This is important because the CSF turns over rapidly, estimated at 1–2 μl min^{-1} in the rat and 0.5 ml min^{-1} in the human[24,25], and thus macromolecules administered directly to the CNS will disappear rapidly. For example, in experimental animals intraventricular administration of albumin has a half-life of 5 h[26]. Consistent with this, we observed that the direct administration of human α1AT to the rat lateral ventricle results in human α1AT moving rapidly throughout the CSF, but disappearing rapidly, with only 3% of the initial level left by 24 h. In contrast, adenovirus-mediated gene transfer to the ependymal cells provides local production, and hence relatively constant levels of the gene product in the CSF for at least 2–4 days. The reason for the decline after 4 days is not obvious, although from our previous experience with adenovirus vectors[17–20], expression is likely dominated by the life-span of the target cells.

The adenovirus-mediated gene transfer to the ependymal cells was not distributed homogeneously, which may reflect the bulk flow of the CSF (which might determine a different degree of exposure of ependymal cells to the vector in different areas of the ventricular system) and/or variable susceptibility of target cells in different regions for adenovirus infection. Despite the inclusion of a nuclear localization signal in the vector, βgal activity was occasionally detected in the cytoplasm of ependymal cells from animals receiving intraventricular Ad.RSVβgal; this phenomenon likely results from diffusion of the βgal in the cytoplasm, or from the β-galactosidase produced in the cytoplasm prior to its transport to the nucleus and is consistent with similar results obtained in other organs[19,20,27].

While we expected ependymal cells to be good targets for the adenovirus vectors because of their epithelial cell origin, there was no *a priori* reason to expect the same for the parenchymal cells of the CNS. However, one of the appealing aspects of adenoviruses for gene transfer is that they are capable of transferring and expressing an exogenous gene *in vivo* in a variety of terminally differentiated or slowly proliferating cells[17–27], characteristics of parenchymal cells of the CNS. The observation of parenchymal cell gene expression in the globus pallidus and striatum and tract posterior to this region following direct injection of the Ad.RSVβgal vector is consistent with this idea. While identification of the cell population targeted by Ad-mediated gene transfer in the brain parenchyma would require the use of specific markers, the morphology of the targeted cells and their location suggest that both neurons and glial elements are likely targeted by the adenovirus vector injection.

The potential use of replication-deficient adenovirus vectors for gene therapy of the CNS is supported by several safety concepts. First, there is no proven association of malignancy with adenovirus infection in humans[28]. Furthermore, type 5 adenovirus (the type used for the construction of the vectors used in this study), is categorized in subgroup C, a subgroup that is nontumorogenic in experimental animals, and there is extensive clinical experience regarding the administratio of live adenoviruses vaccines in humans with no report of association with malignancy[18,19]. Second, it is unlikel that these vectors will adversely harm the target cells, sinc the toxic effects of replication competent adenovirus ar associated with the expression of early viral genes. Th cellular toxicity of adenovirus vectors administrated t the CNS is not known, however the use of replicatio deficient vectors deleted in the E1 region and thus defectiv for early viral genes expression, should minimiz cytotoxicity[28].

One caveat to the effective use of adenovirus-mediate gene transfer to CNS is the possible host immune respons to the vector. This response is not known fo administration to the CNS. However, the immun response to these adenoviruses has been evaluated i animals in different organs such as lungs and peritoneum In these models, there is a humoral immune response t the vector, which may, or may not, interfere with repeate administration, depending on the organ and time betwee the administration of the vector.

While the efficiency and the stability of adenovirus mediated gene transfer remains to be established, th observations in the present study expand the potentia usefulness of adenovirus vectors for CNS gene transfe applications not only involving the CSF, but also to direc delivery to the cells in the brain parenchyma.

Methodology

Adenovirus vectors. The recombinant adenoviral (Ad) vectors use are based on the Ad type 5 (ref. 28), in which the left end of E1 an a portion of the E3 regions are deleted, and a cassette containing recombinant exogenous gene and promoter is inserted in place the E1 deletion. Two different vectors were used: Ad.RSVβga containing the *E. coli lacZ* reporter gene, uses the long termin repeat of the Rous sarcoma virus as a promoter followed by the SV4 large T antigen nuclear localization signal and the *E. coli lac* gene[19,20,27]. Ad-α1AT, containing the human α1AT cDNA, uses th Ad2 major late promoter followed by the human α1AT cDNA Adenovirus vectors were prepared, purified and titered described[17,18]. The purified Ad.RSVβgal preparation was evaluate using a colorimetric analysis for βgal with 2-nitrophenyl-β-D galactopyranoside (ONPG) as a substrate and detection at 420 nm ensure there was no contaminating βgal activity[29]. The purifie Ad-α1AT preparation was evaluated by enzyme-linke immunosorbent assay (ELISA) to ensure there was no contaminati human α1AT[17].

In vivo CNS gene transfer. The experimental animals were Sprague Dawley female rats (250–350 g; 2 months old) prepared by chron cannulation of the left lateral ventricle (Zivic-Miller Laboratorie Inc., Allison Park, PA). The animals were anaesthetized with intraperitoneal injection of a mixture of ketamine-Cl (5 μg g^{-1}) an xylazine (1 μg g^{-1}). Ad.RSVβgal [5×10^8 plaque forming units (pfu) 50 μl of vector vehicle (10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 10 glycerol)] were administered via the ventricular catheter over a 5 m interval. As controls, animals received via the ventricular cathet phosphate buffered saline (PBS) pH 7.4, or an equivalent amount Ad-α1AT. To evaluate the consequences of direct intracerebr administration of an Ad vector, Ad.RSVβgal was administrated uncannulated rats by stereotactic injection in the right globus pallid and in part of the striatum (10^8 pfu in 10–50 μl vector vehicle), usi a 100 μl Hamilton syringe[30]. The injections were made over a 60 m interval, the needle was kept in place for another 2 min, and th removed. As a control, the same animals received intracerebral PI in the area of the left globus pallidus and striatum. Stereotac coordinates were determined from a stereotactic atlas of the adult brain[30].

Evaluation of gene transfer and expression. To evaluate *in vivo* ge transfer and expression at the histologic level, 4 d after *in vi*

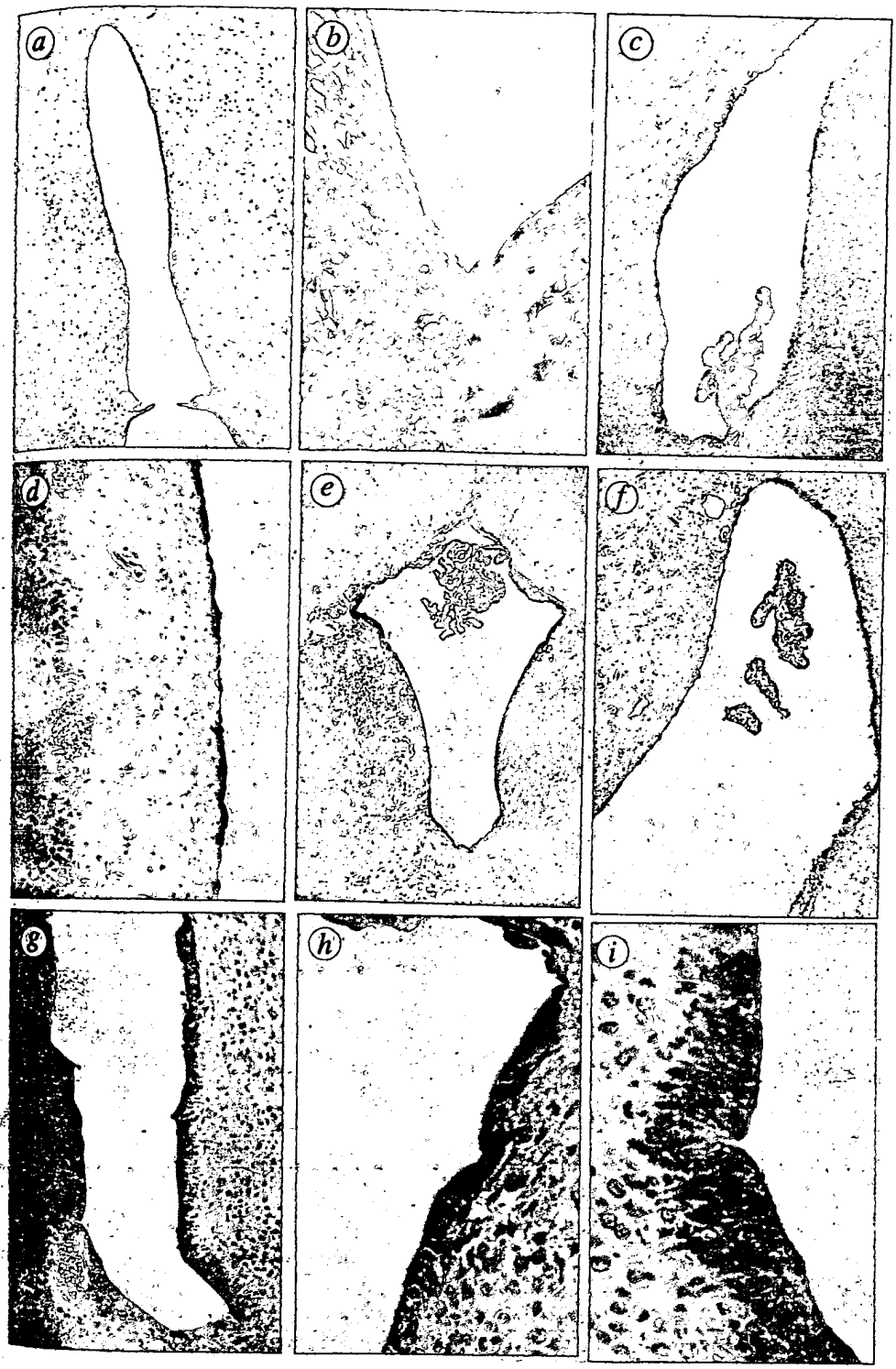


Fig. 2 Expression of β-galactosidase activity in ventricular ependymal cells in brains of rats 4 days after *in vivo* intraventricular administration of Ad.RSVβgal. Control animals received phosphate buffered saline. All sections were stained with the X-Gal reagent and counterstained with nuclear fast red. The blue colour indicates the cells expressing β-galactosidase activity. *a*, Third ventricle, control animal, 50×. *b*, Third ventricle, control, 400×. *c*, Lateral ventricle, Ad.RSVβgal, 100×. Note that only rare cells of the choroid plexus express βgal. *d*, Lateral ventricle, Ad.RSVβgal, 200×. *e*, Third ventricle, Ad.RSVβgal, 100×. Ependymal cells are expressing βgal, but not cells of the choroid plexus. *f*, Fourth ventricle, Ad.RSVβgal, 100×. *g*, Third ventricle, Ad.RSVβgal, 100×. *h,i*, High power view of third ventricle, Ad.RSVβgal, 630×. Note in *g-i*, in the region of the third ventricle shown, cells are pseudostratified.

administration of the Ad.RSVβgal vector (or the controls PBS or Ad-α1AT), the animals were anaesthetized by intraperitoneal injection of pentobarbital (20 μg g^{-1}) and perfused via the heart with cold fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS). The brain was then removed and postfixed in the same fixing solution (90 min, 4 °C). The brain was cut in 4 mm thick coronal sections using a rodent brain matrix (Zivic-Miller Laboratories Inc., Allison Park, PA) and similarly postfixed (30 min, 4 °C). Samples were then washed with PBS, and stained by immersion in 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe_3(CN)_6$ (Sigma) and 2 mM $MgCl_2$ (Mallinckrodt) in PBS containing 0.5 mg ml^{-1} of the X-Gal stain [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim) dissolved in N,N-dimethylformamide (Sigma) at 20 mg ml^{-1}, prior to dilution into the reaction mixture] for 4 h, 37 °C[29,31]. Tissues were identified as positive for βgal activity by the blue stain of the X-Gal reaction. After staining and photography, samples were postfixed in the same fixative, embedded in paraffin, cut into 5 μm sections, and counterstained with nuclear fast red. Cells were considered as positive for the expression of the *lacZ* product if they exhibited the characteristic nuclear/perinuclear βgal blue colour, with or without cytoplasmic staining.

To evaluate *in vivo* Ad-mediated gene transfer and expression of a secreted protein, the amount of human α1AT in the CSF was determined using an ELISA capable of specifically detecting ≥ 3 ng ml^{-1} of human α1AT[17]. To recover the CSF, the animals were anesthetized as described above and the fluid was collected from the fourth

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ventricle using a 1 ml syringe and a 30 gauge needle. The CSF of uninfected animals and animals that received intraventricular Ad-α1AT, PBS, or Ad.RSVβgal were evaluated 0, 2, 4, and 6 days after injection. For comparison, to determine the clearance of human α1AT in the CSF, human α1AT (15–150 μg in 50 μl) were administered via the catheter in the lateral ventricle, and human α1AT was measured by ELISA in the CSF at 0, 10 min, 1, 3, 6, 9 and 24 h.

Statistical evaluation. The α1AT-related data are expressed as mean ± standard error of the mean.

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